

**PCT**

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> A61K 37/00, C07K 13/00 C12N 5/10, 5/12, 15/62 C12N 15/79, G01N 33/53	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/11013</b> <b>(43) International Publication Date:</b> 26 May 1994 (26.05.94)
<b>(21) International Application Number:</b> PCT/US93/10931 <b>(22) International Filing Date:</b> 11 November 1993 (11.11.93)  <b>(30) Priority data:</b> 975,839 13 November 1992 (13.11.92) US  <b>(71) Applicant:</b> DUKE UNIVERSITY [US/US]; Erwin Road, Durham, NC 27706 (US).  <b>(72) Inventors:</b> KANE, William, H. ; 3510 Lochnora Parkway, Durham, NC 27705 (US). ORTEL, Thomas, L. ; 5 Lans- gate Court, Durham, NC 27713 (US).  <b>(74) Agents:</b> SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> CHIMERIC BLOOD COAGULATION PROTEINS  <b>(57) Abstract</b>  Chimeric blood coagulation proteins are disclosed. The proteins are (i) coagulation factor V in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor VIII; or (ii) coagulation factor VIII in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor V. The chimeric proteins are useful for diagnostic purposes in epitope mapping and for therapeutic purposes in facilitating blood coagulations in patients in need of such treatment.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## CHIMERIC BLOOD COAGULATION PROTEINS

This invention was made with Government Support under Grant Number RO1 HL43106 from the National Institutes of Health. The Government has certain rights to this invention.

5

### Field of the Invention

This invention relates to blood coagulation proteins, and particularly relates to Factor VIII and Factor V.

### Background of the Invention

10

Approximately 10-20% of hemophiliacs who receive multiple factor VIII treatments develop alloantibodies that inactivate factor VIII. The development of such factor VIII inhibitors is a serious complication which can result in major bleeding episodes that are difficult to treat. Current therapies include high dose human or porcine factor VIII, steroids, intravenous immune globulin, plasmapheresis, and recombinant factor VIIa. Similar problems may occur in patients undergoing treatment for factor V deficiency. Despite these interventions, many patients require large amounts of blood products during bleeding episodes. Accordingly, there is a continued

15

20

-2-

need for new ways of diagnosing and combating the development of inhibitory alloantibodies in patients undergoing treatment with blood coagulation proteins.

D. Scandella et al., *Blood* 74, 1618 (1989),  
5 describe an *E. coli* expression system for the epitope mapping of factor VIII inhibitors.

D. Scandella et al., *Thromb. Haemost.* 67, 665  
(1992) describe a baculovirus expression system for  
epitope mapping of factor VIII with recombinant factor  
10 VIII peptides.

U.S. Patent No. 5,004,803 to R. Kaufman and  
D. Pittman describe recombinant DNA coding for factor  
VIII in which its B domain is replaced with the B  
domain peptide sequence of factor V. This reference is  
15 concerned with obviating problems in the secretion of  
factor VIII, and not with the active forms of the blood  
coagulation protein. Note that the sequences of human  
factor V and VIII are about 40% identical except in the  
B domain, where there is little homology. See, e.g.,  
20 J. Toole et al. *Nature* 312, 342 (1984); G. Vehar et  
al., *Nature* 312, 337 (1984).

#### Summary of the Invention

Chimeric blood coagulation proteins are  
disclosed herein. The chimeric proteins are selected  
25 from the group consisting of:

coagulation factor V in which at least  
one A3, C1 or C2 domain exon thereof is  
replaced with the homologous exon of  
coagulation factor VIII; and

30 coagulation factor VIII in which at  
least one A3, C1 or C2 domain exon thereof is  
replaced with the homologous exon of  
coagulation factor V.

Chimeric proteins of the instant invention  
35 are useful for diagnostic purposes to epitope map  
alloantibody inhibitors in a patient undergoing

-3-

treatment with a blood coagulation protein to facilitate the design and implementation of a specific therapy based on the inhibitor specificity.

Chimeric proteins of the present invention  
5 are also useful as therapeutic agents in patients with inhibitors which interact with the replaced epitopes of the chimeric blood coagulation protein.

The foregoing and other objects and aspects of the present invention are explained in detail in the  
10 drawings herein and the specification set forth below.

#### Brief Description of the Drawings

The Figure provides a comparison of the domain structures and exon-intron structures for the factor V and factor VIII proteins. The boxes represent  
15 the domain structures for factor V and factor VIII. The identities of the A1, A2, B, A3, C1, and C2 domains are indicated by the letters. The arrows indicate the location of introns which are removed from the mature mRNA. The number of each intron in the gene for factor  
20 V or factor VIII is indicated. Exons 1-25 of the Factor V protein and exons 1-26 of the factor VIII protein are defined by the regions between the arrows, with exons occurring sequentially from left to right.

#### Detailed Description of the Invention

25 Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are presented herein by single strand only, in the 5' to 3'  
30 direction, from left to right.

As noted above, the Figure (adapted from L. Cripe et al., *Biochem.* 31, 3777 (1992)), provides a comparison of the domain structures and exon-intron structures for the factor V and factor VIII proteins.

-4-

The boxes represent the domain structures for factor V and factor VIII. The identities of the A1, A2, B, A3, C1, and C2 domains are indicated by the letters and correspond to the amino acid sequences described in W. Kane and E. Davie, *Blood* 71, 539 (1988). The DNA sequences for human factor VIII and human factor V, the locations of introns and exons, and the sequences of the intron-exon junctions, are known. See, e.g., L. Cripe et al., *Biochem.* 31, 3777 (1992). The arrows in the Figure indicate the location of introns which are removed from the mature mRNA. The number of each intron in the gene for factor V or factor VIII is indicated. Exons 1-25 of the Factor V protein and exons 1-26 of the factor VIII protein are defined by the regions between the arrows, with exons occurring sequentially from left to right (5' to 3'). Those skilled in the art will appreciate that, while the term "exon" is ordinarily used to refer to a region of a genomic DNA which is expressed, the term "exon" is used herein to refer to the portion of the protein encoded by that DNA region.

A single exon or a plurality of exons may be exchanged in the chimera, as illustrated in greater detail below. Where a plurality of exons are exchanged, they may be separate by one or more intervening exons which are not exchanged or, more typically, are adjacent. Where the exchanged exons are adjacent, the number of exons exchanged may be, for example from about 1 to 5 exons, but will more typically be from 2 to 3. As noted above, in many embodiments, a single exon is exchanged.

Some specific examples of chimeric blood coagulation proteins of the present invention are set forth below. Note that factor VIII exons 5 and 6 are sometimes treated as a single exon herein because the two together correspond to exon 5 alone of factor V (due to an additional intron found in factor VIII).

-5-

Note also that, while the chimeric proteins of the present invention are herein described with reference to human blood coagulation proteins, those skilled in the art will appreciate that the invention may be embodied in other mammalian factor V and factor VIII proteins, including the bovine, porcine, and ovine homologs thereof, with variations in intron structure being treated in like manner to the variation in intron structure between human factor V and factor VIII.

10           Factor VIII<sub>(VIII-15;V-14)</sub>;  
               Factor VIII<sub>(VIII-16;V-15)</sub>;  
               Factor VIII<sub>(VIII-17;V-16)</sub>;  
               Factor VIII<sub>(VIII-18;V-17)</sub>;  
               Factor VIII<sub>(VIII-19;V-18)</sub>;  
 15           Factor VIII<sub>(VIII-20;V-19)</sub>;  
               Factor VIII<sub>(VIII-21;V-20)</sub>;  
               Factor VIII<sub>(VIII-22;V-21)</sub>;  
               Factor VIII<sub>(VIII-23;V-22)</sub>;  
               Factor VIII<sub>(VIII-24;V-23)</sub>;  
 20           Factor VIII<sub>(VIII-25;V-24)</sub>;  
               Factor VIII<sub>(VIII-26;V-25)</sub>;  
               Factor V<sub>(V-14;VIII-15)</sub>;  
               Factor V<sub>(V-15;VIII-16)</sub>;  
               Factor V<sub>(V-16;VIII-17)</sub>;  
 25           Factor V<sub>(V-17;VIII-18)</sub>;  
               Factor V<sub>(V-18;VIII-19)</sub>;  
               Factor V<sub>(V-19;VIII-20)</sub>;  
               Factor V<sub>(V-20;VIII-21)</sub>;  
               Factor V<sub>(V-21;VIII-22)</sub>;  
 30           Factor V<sub>(V-22;VIII-23)</sub>;  
               Factor V<sub>(V-23;VIII-24)</sub>;  
               Factor V<sub>(V-24;VIII-25)</sub>; and  
               Factor V<sub>(V-25;VIII-26)</sub>.

In addition, examples of chimeric blood coagulation proteins of the present invention in which two adjacent exons in the A3, C1, or C2 domains are exchanged include:

-6-

- Factor VIII<sub>(VIII-15,16;V-14,15)</sub> ;  
 Factor VIII<sub>(VIII-16,17;V-15,16)</sub> ;  
 Factor VIII<sub>(VIII-17,18;V-16,17)</sub> ;  
 Factor V<sub>(V-14,15;VIII-15,16)</sub> ;  
 5 Factor V<sub>(V-15,16;VIII-16,17)</sub> ;  
 Factor V<sub>(V-16,17;VIII-17,18)</sub> ;  
 Factor VIII<sub>(VIII-20,21;V-19,20)</sub> ;  
 Factor VIII<sub>(VIII-21,22;V-20,21)</sub> ;  
 Factor VIII<sub>(VIII-22,23;V-21,22)</sub> ;  
 10 Factor VIII<sub>(VIII-23,24;V-22,23)</sub> ;  
 Factor VIII<sub>(VIII-24,25;V-23,24)</sub> ;  
 Factor VIII<sub>(VIII-25,26;V-24,25)</sub> ;  
 Factor V<sub>(V-19,20;VIII-20,21)</sub> ;  
 Factor V<sub>(V-20,21;VIII-21,22)</sub> ;  
 15 Factor V<sub>(V-21,22;VIII-22,23)</sub> ;  
 Factor V<sub>(V-22,23;VIII-23,24)</sub> ;  
 Factor V<sub>(V-23,24;VIII-24,25)</sub> ; and  
 Factor V<sub>(V-24,25;VIII-25,26)</sub> .

Some examples of chimeras in which three,  
 20 four, and five adjacent exons are exchanged are the  
 following:

- Factor VIII<sub>(VIII-20,21,22;V-19,20,21)</sub> ;  
 Factor VIII<sub>(VIII-20,21,22,23;V-19,20,21,22)</sub> ; and  
 Factor VIII<sub>(VIII-20,21,22,23,24;V-19,20,21,22,23)</sub> .  
 25 The foregoing chimeric proteins may be made  
 in accordance with techniques known in the art. The  
 production of recombinant DNA, vectors, host cells, and  
 proteins by genetic engineering techniques is well  
 known. See, e.g., U.S. Patent No. 4,761,371 to Bell et  
 30 al. at Col. 6 line 3 to Col. 9 line 65; U.S. Patent No.  
 4,877,729 to Clark et al. at Col. 4 line 38 to Col. 7  
 line 6; U.S. Patent No. 4,912,038 to Schilling at Col.  
 3 line 26 to Col. 14 line 12; and U.S. Patent No.  
 4,879,224 to Wallner at Col. 6 line 8 to Col. 8 line  
 35 59.

DNA sequences encoding chimeric proteins may  
 be recovered by use of the polymerase chain reaction



-7-

(PCR) procedure and splicing by overlap extension (SOE), as is known in the art. See U.S. Patents Nos. 4,683,195 to Mullis et al. and 4,683,202 to Mullis.

The chimeric proteins may be synthesized in  
5 host cells transformed with vectors containing DNA encoding the chimeric proteins. A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding the chimeric protein and/or to express DNA which encodes the chimeric  
10 protein. An expression vector is a replicable DNA construct in which a DNA sequence encoding the chimeric protein is operably linked to suitable control sequences capable of effecting the expression of the chimeric protein in a suitable host. The need for such  
15 control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA  
20 ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of  
25 replication, and a selection gene to facilitate recognition of transformants.

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e.,  
30 fragments integratable into the host genome by homologous recombination). The vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control  
35 sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or

-8-

transfected with the chimeric protein vectors constructed using recombinant DNA techniques. Transformed host cells ordinarily express the chimeric protein, but host cells transformed for purposes of cloning or amplifying the chimeric protein DNA need not express the chimeric protein. When expressed, the chimeric protein will typically be located in the host cell membrane.

DNA regions are operably linked when they are functionally related to each other. For example: a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, operably linked means contiguous and, in the case of leader sequences, contiguous and in reading phase.

Suitable host cells include prokaryotes, yeast cells or higher eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example *Escherichia coli* (*E. coli*) or Bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Exemplary host cells are *E. coli* W3110 (ATCC 27,325), *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* 294 (ATCC 31,446). *Pseudomonas* species, *Bacillus* species, and *Serratia marcesans* are also suitable.

A broad variety of suitable microbial vectors are available. Generally, a microbial vector will contain an origin of replication recognized by the intended host, a promoter which will function in the host and a phenotypic selection gene such as a gene encoding proteins conferring antibiotic resistance or supplying an autotrophic requirement. Similar constructs will be manufactured for other hosts. *E. coli* is typically transformed using pBR322. See Bolivar et al., *Gene* 2, 95 (1977). pBR322 contains

-9-

genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells.

Expression vectors should contain a promoter  
5 which is recognized by the host organism. This generally means a promoter obtained from the intended host. Promoters most commonly used in recombinant microbial expression vectors include the beta-lactamase (penicillinase) and lactose promoter systems (Chang et  
10 al., *Nature* 275, 615 (1978); and Goeddel et al., *Nature* 281, 544 (1979)), a tryptophan (trp) promoter system (Goeddel et al., *Nucleic Acids Res.* 8, 4057 (1980) and EPO App. Publ. No. 36,776) and the tac promoter (H. De Boer et al., *Proc. Natl. Acad. Sci. USA* 80, 21 (1983)).  
15 While these are commonly used, other microbial promoters are suitable. Details concerning nucleotide sequences of many have been published, enabling a skilled worker to operably ligate them to DNA encoding the chimeric protein in plasmid or viral vectors  
20 (Siebenlist et al., *Cell* 20, 269 (1980)). The promoter and Shine-Dalgarno sequence (for prokaryotic host expression) are operably linked to the DNA encoding the chimeric protein, i.e., they are positioned so as to promote transcription of the chimeric protein messenger  
25 RNA from the DNA.

Eukaryotic microbes such as yeast cultures may be transformed with suitable chimeric protein-encoding vectors. See, e.g., U.S. Patent No. 4,745,057. *Saccharomyces cerevisiae* is the most  
30 commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors may contain an origin of replication from the 2 micron yeast plasmid or an autonomously replicating sequence (ARS), a  
35 promoter, DNA encoding the chimeric protein, sequences for polyadenylation and transcription termination, and a selection gene. An exemplary plasmid is YRp7,

-10-

(Stinchcomb et al., *Nature* 282, 39 (1979); Kingsman et al., *Gene* 7, 141 (1979); Tschemper et al., *Gene* 10, 157 (1980)). This plasmid contains the *trp1* gene, which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, *Genetics* 85, 12 (1977)). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255, 2073 (1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7, 149 (1968); and Holland et al., *Biochemistry* 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publ. No. 73,657.

Cultures of cells derived from multicellular organisms are a desirable host for recombinant chimeric protein synthesis. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture, including insect cells. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. See *Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973). Examples of useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and WI138, BHK, COS-7, CV, and MDCK cell lines. Expression vectors for such cells ordinarily include (if

-11-

necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing genomic DNA is used), a  
5 polyadenylation site, and a transcriptional termination sequence.

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by  
10 viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). See, e.g., U.S. Patent No. 4,599,308. The early and late promoters are useful because both are obtained easily from the virus as a fragment which  
15 also contains the SV40 viral origin of replication. See Fiers et al., *Nature* 273, 113 (1978). The vaccinia virus may be used as a vector, as described in the Examples. Further, the chimeric protein promoter, control and/or signal sequences, may also be used,  
20 provided such control sequences are compatible with the host cell chosen.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or  
25 other viral source (e.g. Polyoma, Adenovirus, VSV, or BPV), or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient.

30 Rather than using vectors which contain viral origins of replication, one can transform mammalian cells by the method of cotransformation with a selectable marker and the chimeric protein DNA. An example of a suitable selectable marker is  
35 dihydrofolate reductase (DHFR) or thymidine kinase. See U.S. Pat. No. 4,399,216. Such markers are proteins, generally enzymes, that enable the identification of

-12-

transformant cells, i.e., cells which are competent to take up exogenous DNA. Generally, identification is by survival of transformants in culture medium that is toxic, or from which the cells cannot obtain critical  
5 nutrition without having taken up the marker protein.

Host cells such as insect cells (e.g., cultured *Spodoptera frugiperda* cells) and expression vectors such as the baculovirus expression vector (e.g., vectors derived from *Autographa californica*  
10 MNPV, *Trichoplusia ni* MNPV, *Rachiplusia ou* MNPV, or *Galleria ou* MNPV) may be employed in carrying out the present invention, as described in U.S. Patents Nos. 4,745,051 and 4,879,236 to Smith et al. In general, a baculovirus expression vector comprises a baculovirus  
15 genome containing the gene to be expressed inserted into the polyhedrin gene at a position ranging from the polyhedrin transcriptional start signal to the ATG start site and under the transcriptional control of a baculovirus polyhedrin promoter.

20 The chimeric proteins described herein may be prepared per se or in the form of pharmaceutically acceptable salts thereof. For example, acid addition salts of acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate,  
25 citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfonate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-  
30 hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and  
35 undecanoate. Base salts include ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and

-13-

magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine, lysine, and so forth.

5                   Pharmaceutical formulations of the instant invention comprise the chimeric protein in a pharmaceutically acceptable carrier, such as sterile, pyrogen-free water or sterile pyrogen-free phosphate-buffered saline solution. The chimeric protein is  
10 included in an effective coagulation-promoting amount. The precise amount to be administered to the patient (i.e., a human patient) is determined in a routine manner, and will vary depending on the condition of the subject, route of administration (e.g., intravenous,  
15 subcutaneous, intraperitoneal). In general, for factor VIII, the dosage will range from 5 or 10 to 50, 500 or even 5,000 Units or more per kilogram subject body weight. Dosages for factor V may be the same, although in practice factor V dosage is typically determined by  
20 simply administering a factor V-containing preparation and monitoring the patient for the desired effect.

Diagnostic tests of the present invention may be carried out in accordance with known techniques. Such techniques provide a method of detecting antibody  
25 inhibitors to a blood coagulation protein in a patient (typically a human patient), comprising collecting an antibody-containing biological fluid sample (e.g., blood, blood plasma, or blood serum) from the patient; contacting the biological fluid sample to a chimeric  
30 blood coagulation protein as given herein; and then detecting the formation of a reaction product between said chimeric blood coagulation protein and antibodies in said biological fluid. Any suitable assay format, such as radioimmunoassay (RIA) or enzyme-linked  
35 immunosorbent assay (ELISA) may be employed, in accordance with known techniques. See, e.g., Immunology: Basic Processes, 162-175 (J. Bellanti Ed.

-14-

2d Ed. 1985) (W.B. Saunders Co.). To carry out epitope mapping, the assay is repeated with a biological sample from a single subject a plurality of times (or repetitions) with different chimeric proteins (e.g., a library of chimeric proteins) with each member of the library containing a different, predetermined, epitope (e.g., a different Factor VIII epitope in Factor V for Factor VIII epitope mapping; a different Factor V epitope in a Factor VIII for factor V epitope mapping).

10 Identification of the particular chimera or chimeras with which patient antibodies react enables identification of the epitopes in those chimeras to which the alloantibody or autoantibody inhibitors in the patient are directed.

15 The present invention is explained in greater detail in the following Examples. These examples are for illustrative purposes only, and are not to be taken as limiting of the invention.

#### EXAMPLES

20 *Construction of Light Chain Chimeras* The full-length factor V cDNA in the plasmid pUC 18 and the full-length factor VIII cDNA in the plasmid pCNHS were used for the construction of all mutants. To prepare chimeras that precisely switched exon-size segments of

25 factor VIII cDNA for the corresponding segment of factor V, a combination of restriction fragments and the polymerase chain reaction (PCR) were used, as previously described (Ortel, TL, Devore-Carter, D, Quinn-Allen, MA, and Kane, WH. (1992) "Deletion

30 Analysis of Recombinant Human Factor V. Evidence for a Phosphatidylserine Binding Site in the Second C-Type Domain" J. Biol Chem, 267: 4189-4198). The mutant rHFV<sub>V,e25;VIII,e26</sub>, which has exon 26 of factor VIII substituted for exon 25 of factor V, was constructed as

35 follows. The last exon of factor VIII was amplified using oligonucleotides 1 and 2 (Table 1). This



-15-

fragment was restricted with Nco 1/Sal 1, and inserted with a Bam H1/Nco 1 fragment from factor V (spanning residues 6000 through 6591 of the cDNA) into Bam H1/sal 1 restricted pUC 18. A Bgl 11/Sal 1 fragment, spanning  
5 the entire chimeric C2 domain construct, was excised and subsequently ligated into the shuttle vector pCNVSS rHFV LC that had been restricted with Bgl 11/Sal 1. The resultant construct, containing the single exon switch, was restricted with Sph 1/Sal 1, which released  
10 the entire chimeric light chain constructed from pCNVSS. This chimera was then ligated into pCNHS rHFV that had been cut with Sph 1/Sal 1, replacing the light chain of factor V with the chimeric light chain.

The mutant rHFV<sub>V,e24,25;VIII,e25,26</sub>, which has exons  
15 25 and 26 of factor VIII substituted for exons 24 and 25 of factor V, was constructed using the PCR to splice exon 23 of factor V to exon 25 of factor VIII by overlap extension (SOE) (Ho, SN, Hunt, HD, Horton, RM, Pullen, JK, and Pease, IR. (1989) *Gene*, 77: 51). The  
20 PCR was used to amplify two segments of DNA, one spanning exons 19 through 23 of factor V and one spanning exons 25 and 26 of factor VIII, using oligonucleotides 3 and 4, and 5 and 6, respectively, as primers. The resultant PCR products overlapped by  
25 approximately 30 bp at the 3' end of exon 23 of factor V and the 5' end of exon 25 of factor VIII. These fragments were purified by agarose gel electrophoresis and used as the templates in a second PCR, using oligonucleotides 3 and 67 as primers. The resultant  
30 PCR fragment, spanning approximately 1000 bp, was restricted with Bam H1/Sal 1 and shuttled into pCNVSS rHFV LC and the pCNHS rHFV, as described for rHFV<sub>V,e25;VIII,e26</sub>.

The mutant rHFV<sub>V,C2;VIII,C2</sub>, which contains the  
35 entire C2 domain of factor VIII substituted for the C2 domain of factor V, was constructed by using the PCR with factor V as a template and oligonucleotides 7 and

-16-

8 as primers. This resulted in an amplified C1 domain of factor V that possessed the first fifteen bases of the C2 domain of factor VIII, including an Sph I site. This fragment was restricted with Bam HI/Sph I and was  
5 ligated with an Sph I/Sal I fragment from factor VIII (spanning the C2 domain) into Bam HI/Sal I restricted pUC 18. The fragment was then excised with Bgl II/Sal I and inserted into pCNVSS rHFV LC, as described for rHFV<sub>V,e25;VIII,e26</sub>. Because of the Sph I site in exon 24 of  
10 factor VIII, this chimeric light chain was restricted with Nco I/Sal I and inserted into Nco I/Sal I restricted pCNHS rHFV.

The structures of all factor V chimeras were confirmed by extensive restricting mapping and DNA  
15 sequencing of all segments obtained by the PCR. The cDNA inserts were then excised from the plasmid by Cla I/Sal I and ligated into the expression vector pDX<sub>ECSE</sub>. The resultant expression vectors were obtained from JM109 cells using the alkaline lysis method, as  
20 previously described (Ortel, *supra*).

*Transient Expression of Factor V Chimeras in COS cells* COS-7 cells were transfected with the individual mutant plasmid constructs by calcium phosphate precipitation as described previously (Ortel,  
25 *supra*).

TABLE 1

Mutant	Oligonucleotide	Strand	Sequence	SEQ ID NO
rHFV <sub>V</sub> , e25; VIII, e26	1	+	TCTCCATGGTGGACAAGGTTTTTCAGGGAAATCAAG	SEQ ID NO:1
	2	-	CACAGTCGACTCAGTAGAGGTCCTGTGCCTC	SEQ ID NO:2
rHFV <sub>V</sub> , e24-25; VIII, e25, 25	3	+	AATGCCTGGCAAGCCCAAGGTGAATAATCCAAAAGAG	SEQ ID NO:3
	4	-	CACAGTCGACTCAGTAGAGGTCCTGTGCCTC	SEQ ID NO:4
	5	+	CACAGGTACCTCAGAGACTGTAGGATGCCA	SEQ ID NO:5
	6	-	CTCTTTTGGATTATTCACCTTGGCTTGCCAGGCATT	SEQ ID NO:6
rHRV <sub>V</sub> , c2; VIII, c2	7	+	CACAGGTACCTCAGAGACTGTAGGATGCCA	SEQ ID NO:7
	8	-	CAATGGCATGCTGCAACTATTTACCTCACAAACCTTG	SEQ ID NO:8

-18-

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be  
5 included therein.

-19-

**THAT WHICH IS CLAIMED IS:**

1. A chimeric blood coagulation protein selected from the group consisting of:

coagulation factor V in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor VIII; and

coagulation factor VIII in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor V.

2. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

coagulation factor V in which at least one A3 domain exon thereof is replaced with the homologous A3 domain exon of coagulation factor VIII; and

coagulation factor VIII in which at least one A3 domain exon thereof is replaced with the homologous A3 domain exon of coagulation factor V.

3. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

coagulation factor V in which at least one C1 domain exon thereof is replaced with the homologous C1 domain exon of coagulation factor VIII; and

coagulation factor VIII in which at least one C1 domain exon thereof is replaced with the homologous C1 domain exon of coagulation factor V.

-20-

4. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

5 coagulation factor V in which at least one C2 domain exon thereof is replaced with the homologous C2 domain exon of coagulation factor VIII; and

10 coagulation factor VIII in which at least one C2 domain exon thereof is replaced with the homologous C2 domain exon of coagulation factor V.

5. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

15 coagulation factor V in which from 1 to 5 adjacent exons thereof are replaced with the homologous exons of coagulation factor VIII; and

20 coagulation factor VIII in which from 1 to 5 adjacent exons thereof are replaced with the homologous exons of coagulation factor V.

6. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

25 Factor VIII<sub>(VIII-15;V-14)</sub>;  
Factor VIII<sub>(VIII-16;V-15)</sub>;  
Factor VIII<sub>(VIII-17;V-16)</sub>;  
Factor VIII<sub>(VIII-18;V-17)</sub>;  
Factor VIII<sub>(VIII-19;V-18)</sub>;  
30 Factor VIII<sub>(VIII-20;V-19)</sub>;  
Factor VIII<sub>(VIII-21;V-20)</sub>;  
Factor VIII<sub>(VIII-22;V-21)</sub>;  
Factor VIII<sub>(VIII-23;V-22)</sub>;  
Factor VIII<sub>(VIII-24;V-23)</sub>;  
35 Factor VIII<sub>(VIII-25;V-24)</sub>;

-21-

Factor VIII<sub>(VIII-26;V-25)</sub> ;

Factor V<sub>(V-14;VIII-15)</sub> ;

Factor V<sub>(V-15;VIII-16)</sub> ;

Factor V<sub>(V-16;VIII-17)</sub> ;

5 Factor V<sub>(V-17;VIII-18)</sub> ;

Factor V<sub>(V-18;VIII-19)</sub> ;

Factor V<sub>(V-19;VIII-20)</sub> ;

Factor V<sub>(V-20;VIII-21)</sub> ;

Factor V<sub>(V-21;VIII-22)</sub> ;

10 Factor V<sub>(V-22;VIII-23)</sub> ;

Factor V<sub>(V-23;VIII-24)</sub> ;

Factor V<sub>(V-24;VIII-25)</sub> ;

Factor V<sub>(V-25;VIII-26)</sub> ;

Factor VIII<sub>(VIII-15,16;V-14,15)</sub> ;

15 Factor VIII<sub>(VIII-16,17;V-15,16)</sub> ;

Factor VIII<sub>(VIII-17,18;V-16,17)</sub> ;

Factor V<sub>(V-14,15;VIII-15,16)</sub> ;

Factor V<sub>(V-15,16;VIII-16,17)</sub> ;

Factor V<sub>(V-16,17;VIII-17,18)</sub> ;

20 Factor VIII<sub>(VIII-20,21;V-19,20)</sub> ;

Factor VIII<sub>(VIII-21,22;V-20,21)</sub> ;

Factor VIII<sub>(VIII-22,23;V-21,22)</sub> ;

Factor VIII<sub>(VIII-23,24;V-22,23)</sub> ;

Factor VIII<sub>(VIII-24,25;V-23,24)</sub> ;

25 Factor VIII<sub>(VIII-25,26;V-24,25)</sub> ;

Factor V<sub>(V-19,20;VIII-20,21)</sub> ;

Factor V<sub>(V-20,21;VIII-21,22)</sub> ;

Factor V<sub>(V-21,22;VIII-22,23)</sub> ;

Factor V<sub>(V-22,23;VIII-23,24)</sub> ;

30 Factor V<sub>(V-23,24;VIII-24,25)</sub> ;

Factor V<sub>(V-24,25;VIII-25,26)</sub> ;

Factor VIII<sub>(VIII-20,21,22;V-19,20,21)</sub> ;

Factor VIII<sub>(VIII-20,21,22,23;V-19,20,21,22)</sub> ; and

Factor VIII<sub>(VIII-20,21,22,23,24;V-19,20,21,22,23)</sub> •

-22-

7. A chimeric blood coagulation protein according to claim 1 selected from the group consisting of:

- Factor VIII<sub>(VIII-20;V-19)</sub> ;
- 5 Factor VIII<sub>(VIII-21;V-20)</sub> ;
- Factor VIII<sub>(VIII-22;V-21)</sub> ;
- Factor VIII<sub>(VIII-23;V-22)</sub> ;
- Factor VIII<sub>(VIII-24;V-23)</sub> ;
- Factor VIII<sub>(VIII-25;V-24)</sub> ;
- 10 Factor VIII<sub>(VIII-26;V-25)</sub> ;
- Factor V<sub>(V-19;VIII-20)</sub> ;
- Factor V<sub>(V-20;VIII-21)</sub> ;
- Factor V<sub>(V-21;VIII-22)</sub> ;
- Factor V<sub>(V-22;VIII-23)</sub> ;
- 15 Factor V<sub>(V-23;VIII-24)</sub> ;
- Factor V<sub>(V-24;VIII-25)</sub> ;
- Factor V<sub>(V-25;VIII-26)</sub> ;
- Factor VIII<sub>(VIII-20,21;V-19,20)</sub> ;
- Factor VIII<sub>(VIII-21,22;V-20,21)</sub> ;
- 20 Factor VIII<sub>(VIII-22,23;V-21,22)</sub> ;
- Factor VIII<sub>(VIII-23,24;V-22,23)</sub> ;
- Factor VIII<sub>(VIII-24,25;V-23,24)</sub> ;
- Factor VIII<sub>(VIII-25,26;V-24,25)</sub> ;
- Factor V<sub>(V-19,20;VIII-20,21)</sub> ;
- 25 Factor V<sub>(V-20,21;VIII-21,22)</sub> ;
- Factor V<sub>(V-21,22;VIII-22,23)</sub> ;
- Factor V<sub>(V-22,23;VIII-23,24)</sub> ;
- Factor V<sub>(V-23,24;VIII-24,25)</sub> ; and
- Factor V<sub>(V-24,25;VIII-25,26)</sub> .

30 8. An isolated DNA encoding a chimeric blood coagulation protein according to claim 1.

9. A vector comprising isolated DNA according to claim 8.



-23-

10. A host cell containing a vector according to claim 9.

11. A host cell containing a vector according to claim 9 and capable of expressing the  
5 same.

12. A method of detecting antibody inhibitors to a blood coagulation protein in a patient, comprising:

collecting an antibody-containing biological  
10 fluid sample from said patient;

contacting said biological fluid sample to a chimeric blood coagulation protein selected from the group consisting of:

coagulation factor V in which at  
15 least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor VIII; and  
coagulation factor VIII in which at  
least one A3, C1 or C2 domain exon  
20 thereof is replaced with the homologous exon of coagulation factor V; and then  
detecting the formation of a reaction product between said chimeric blood coagulation protein and antibodies in said biological fluid.

13. A method according to claim 12, wherein said biological fluid is selected from the group consisting of blood, blood plasma, and blood serum.

14. A pharmaceutical formulation comprising an effective coagulation-enhancing amount of a chimeric  
30 blood coagulation protein in a pharmaceutically acceptable carrier, said chimeric blood coagulation protein selected from the group consisting of:

-24-

coagulation factor V in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor VIII; and

5

coagulation factor VIII in which at least one A3, C1 or C2 domain exon thereof is replaced with the homologous exon of coagulation factor V.

### Factor V

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
A1			B									A3			C1			C2					

### Factor VIII

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
A1			A2									B			A3			C1			C2			

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/10931

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 435/7.1, 320.1, 240.2; 514/8, 12; 530/380, 383; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 320.1, 240.2; 514/8, 12; 530/380, 383; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, BIOSIS, APS, WORLD PATENTS INDEX

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,849,407 (Murray et al) 18 July 1989, see the entire document.	1-14
<del>X</del> Y	US, A, 5,004,803 (Kaufman et al) 02 April 1991, see the entire document.	<del>1-4,8-11,14</del> 5-7,12-13
Y	Blood, Volume 71, No. 3, issued March 1988, W. H. Kane et al, "Blood Coagulation Factors V and VIII: Structural and Functional Similarities and Their Relationship to Hemorrhagic and Thrombotic Disorders", pages 539-555, see pages 541 and 542.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A document defining the general state of the art which is not considered to be part of particular relevance	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E earlier document published on or after the international filing date	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* Z	document member of the same patent family
* O document referring to an oral disclosure, use, exhibition or other means		
* P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

23 December 1993

Date of mailing of the international search report

08 FEB 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

R. KEITH BAKER



Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/10931

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemistry, Volume 31, No. 15, issued 1992, L. D. Cripe et al, "Structure of the Gene for Human Coagulation Factor V", pages 3777-3785, see pages 3782 and 3783.	1-14
Y	Blood, Volume 65, No. 4, issued April 1985, H. C. Chiu et al, "Immune Complexes Containing Factor V in a Patient With an Acquired Neutralizing Antibody", pages 810-818, see the Abstract.	1-14
Y	Blood, Volume 74, No. 5, issued October 1989, D. Scandella et al, "Localization of Epitopes for Human Factor VIII Inhibitor Antibodies by Immunoblotting and Antibody Neutralization", pages 1618-1626, see the Abstract.	1-14
Y	Thrombosis and Haemostasis, Volume 67, issued 1992, D. Scandella et al, "A Soluble Recombinant Factor VIII Fragment Containing the A2 Domain Binds to Some Human Anti-Factor VIII Antibodies that are not Detected by Immunoblotting", pages 665-671, see the Abstract.	12-13
Y	Nature, Volume 312, issued 22 November 1984, Toole et al, "Molecular Cloning of a cDNA Encoding Human Antihaemophilic Factor" pages 342-347, see entire document.	1-14

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/10931

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 37/00; C07K 13/00; C12N 5/10, 5/12, 15/62, 15/79; G01N 33/53

Form PCT/ISA/210 (extra sheet)(July 1992)\*